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DESCRIPTION

HIGHLY PRODUCTIVE α-AMYLASES

Technical Field

The present invention relates to mutant α-amylases having improved productivity.

Background Art

α-Amylases [EC.3.2.1.1.] have been used in a wide range of industrial fields such as starch industry, brewing industry, fiber industry, pharmaceutical industry and food industry. Among them, those capable of degrading starches at high random are suited for detergents. Conventionally known as such are, as well as α-amylases derived from *Bacillus licheniformis*, liquefying alkaline α-amylases derived from the alkaliphilic strain *Bacillus* sp. KSM-AP1378 (FERM BP-3048) (WO94/26881) and improved enzymes having improved heat resistance and oxidant resistance (WO98/44126).

The present inventors have recently found liquefying alkaline α-amylases derived from the alkaliphilic strain *Bacillus* sp. KSM-K38 (FERM BP-6946) and having chelating-agent- and oxidation-resistance (Japanese Patent Application No. Hei 10-362487, Japanese Patent Application

No. Hei 10-362488); and improved enzymes having improved heat resistance (Japanese Patent Application No. Hei 11-163569).

In addition to such properties, enzymes for detergents are required to have high productivity in consideration of their industrial production. Although various trials have been made to improve the heat resistance or oxidant resistance of α-amylases for detergent by using protein engineering technique, neither improvement of productivity has been considered sufficiently nor an attempt of production increase by mutation of a structural gene has been reported yet.

An object of the present invention is to provide mutant α-amylases having excellent productivity.

Disclosure of the Invention

The present inventors introduced, in microorganisms, mutant α-amylase structural gene constructed by site-directed mutagenesis and evaluated productivity of α-amylases. As a result, it has been found that since an α-amylase gene has a site taking part in the improvement of productivity, introduction, into a microorganism, of a recombinant gene having this site mutated makes it possible to produce α-amylases having drastically improved productivity.

In one aspect of the present invention, there is thus provided a mutant α-amylase which is derived from an α-amylase having an amino acid sequence represented by SEQ ID No. 1 or showing at least 60% homology thereto by substitution or deletion of at least one amino acid residue corresponding to any one of Pro_{18}, Gln_{86}, Glu_{130}, Asn_{154}, Arg_{171}, Ala_{186}, Glu_{212}, Val_{222}, Tyr_{243}, Pro_{260}, Lys_{269}, Glu_{276}, Asn_{277}, Arg_{310}, Glu_{360}, Gln_{391}, Trp_{439}, Lys_{444}, Asn_{471} and Gly_{476} of the amino acid sequence.

In another aspect of the present invention, there is also provided a mutant α-amylase derived from an α-amylase having an amino acid sequence represented by SEQ ID No. 2 or showing at least 60% homology thereto by substitution or deletion of at least one amino acid residue corresponding to any one of Asp_{128}, Gly_{140}, Ser_{144}, Arg_{168}, Asn_{181}, Glu_{207}, Phe_{272}, Ser_{375}, Trp_{434} and Glu_{466} of the amino acid sequence.

In a further aspect of the present invention, there is also provided a gene encoding this mutant α-amylase, a vector containing the gene, a cell transformed with the vector and a production method of a mutant α-amylase which comprises cultivating the transformed cell.

In a still further aspect of the present invention, there is also provided a detergent composition containing this mutant α-amylase.

Brief Description of the Drawings

FIG. 1 illustrates a method of constructing a recombinant plasmid for production of an α-amylase derived from the strain KSM-1378 or KSM-K38.

FIG. 2 is a schematic view illustrating a method of introducing a mutation into an α-amylase gene derived from the strain KSM-1378 or KSM-K38.

Best Mode for Carrying out the Invention

The term "highly productive mutant α-amylase" as used herein means an α-amylase whose yield is increased, upon production of it by cultivating a recombinant microorganism, by at least 5%, preferably at least 10%, more preferably at least 20% compared with that before mutation.

The mutant α-amylase of the present invention is constructed so that out of amino acids constituting the α-amylase, the amino acid residues taking part in the productivity are substituted with another amino acid residues or deleted. Examples of the α-amylase usable here include liquefying α-amylases derived from *Bacillus. amyloliquefaciens* or *Bacillus. licheniformis* and liquefying alkaline α-amylases derived from alkaliphilic microorganisms belonging to the *Bacillus* sp., of which α-

amylases having an amino acid sequence represented by SEQ ID No. 1 or SEQ ID No. 2 and α-amylases having at least 60% homology to the above-described amino acid sequence are preferred.

Examples of the α-amylase having the amino acid sequence represented by SEQ ID No. 1 or α-amylase having at least 60% homology thereto include liquefying alkaline α-amylases derived from the strain *Bacillus* sp. KSM-AP1378 (FERM BP-3048) (Japanese Patent Application Laid-Open No. Hei 8-336392) and improved enzymes of the above-described one in heat resistance and oxidant resistance which are constructed by protein engineering technique (WO98/44126).

Examples of the α-amylase having the amino acid sequence represented by SEQ ID No. 2 or having at least 60% homology thereto include liquefying alkaline α-amylases derived from the strain *Bacillus* sp. KSM-K38 (FERM BP-6946) and improved enzymes of the above-described one in heat resistance which are constructed by protein engineering technique (Japanese Patent Application No. Hei 11-163569).

The homology of an amino acid sequence is calculated by Lipman-Pearson method (Science, **227**, 1435(1985)).

The mutant α-amylase of the present invention can be obtained first by cloning, from a microorganism producing an α-amylase, a gene encoding the α-amylase. For this

purpose, ordinarily employed gene recombinant technique, for example, the method as described in Japanese Patent Application Laid-Open No. Hei 8-336392 may be employed. Examples of the gene usable here include that represented by SEQ ID No. 3 or SEQ ID No. 4 which encodes the amino acid sequence represented by SEQ ID No. 1 or SEQ ID No. 2. Mutant genes derived from the above-described ones and having improved heat resistance and oxidant resistance are also usable.

For mutation of the gene thus obtained by cloning, any site-directed mutagenesis ordinarily employed can be adopted. For example, mutation can be conducted using a "Site-Directed Mutagenesis System Mutan-Super Express Km" kit (product of Takara Shuzo Co., Ltd.).

Mutation for obtaining highly productive α-amylases of the invention can be attained, for example, by substitution or deletion, in an α-amylase having an amino acid sequence represented by SEQ ID No. 1 or having at least 60% homology thereto, of at least one amino acid residue corresponding to any one of Pro_{18}, Gln_{86}, Glu_{130}, Asn_{154}, Arg_{171}, Ala_{186}, Glu_{212}, Val_{222}, Tyr_{243}, Pro_{260}, Lys_{269}, Glu_{276}, Asn_{277}, Arg_{310}, Glu_{360}, Gln_{391}, Trp_{439}, Lys_{444}, Asn_{471} and Gly_{476} of the amino acid sequence; or by substitution or deletion, in another α-amylase having an amino acid sequence represented by SEQ ID No. 2 or having at least 60% homology thereto, of at

least one amino acid residue corresponding to any one of Asp_{128}, Gly_{140}, Ser_{144}, Arg_{168}, Asn_{181}, Glu_{207}, Phe_{272}, Ser_{375}, Trp_{434} and Glu_{466} of the amino acid sequence. Preferred mutations include, in the amino acid sequence of SEQ ID No. 1, substitution of the amino acid residue corresponding to Pro_{18} with Ser or Thr, the amino acid residue corresponding to Gln_{86} with Glu, the amino acid residue corresponding to Glu_{130} with Val or Gln, the amino acid residue corresponding to Asn_{154} with Asp, the amino acid residue corresponding to Arg_{171} with Cys or Gln, the amino acid residue corresponding to Ala_{186} with Val or Asn, the amino acid residue corresponding to Glu_{212} with Asp, the amino acid residue corresponding to Val_{222} with Glu, the amino acid residue corresponding to Tyr_{243} with Cys or Ser, the amino acid residue corresponding to Pro_{260} with Glu, the amino acid residue corresponding to Lys_{269} with Gln, the amino acid residue corresponding to Glu_{276} with His, the amino acid residue corresponding to Asn_{277} with Ser or Phe, the amino acid residue corresponding to Arg_{310} with Ala, the amino acid residue corresponding to Glu_{360} with Gln, the amino acid residue corresponding to Gln_{391} with Glu, the amino acid residue corresponding to Trp_{439} with Arg, the amino acid residue corresponding to Lys_{444} with Arg, the amino acid residue corresponding to Asn_{471} with Asp or Glu, or the amino acid residue corresponding to Gly_{476} with Asp;

or substitution, in the amino acid sequence of SEQ ID

No. 2, of the amino acid residue corresponding to Asp_{128} with Val or Gln, the amino acid residue corresponding to Gly_{140} with Ser, the amino acid residue corresponding to Ser_{144} with Pro, the amino acid residue corresponding to Arg_{168} with Gln, the amino acid residue corresponding to Gln_{181} with Val, the amino acid residue corresponding to Glu_{270} with Asp, the amino acid residue corresponding to Phe_{272} with Ser, the amino acid residue corresponding to Ser_{375} with Pro, the amino acid residue corresponding to Trp_{434} with Arg or the amino acid residue corresponding to Glu_{466} with Asp.

Among the mutations of the amino acid sequence of SEQ ID No. 1, those by substitution of the amino acid residue corresponding to Gln_{86} with Glu, the amino acid residue corresponding to Glu_{130} with Val or Gln, the amino acid residue corresponding to Ala_{186} with Asn, the amino acid residue corresponding to Tyr_{243} with Ser, the amino acid residue corresponding to Pro_{260} with Glu, the amino acid residue corresponding to Lys_{269} with Gln, the amino acid residue corresponding to Asn_{277} with Phe and the amino acid residue corresponding to Asn_{471} with Asp or Glu can bring about improvement in solubility of the α-amylase in a culture medium or desalted and concentrated solution thereof. More specifically, the above-described mutations make it possible to improve the residual activity of the α-

amylase in the supernatant after storage at 4°C for one week in a desalted and concentrated solution by at least 5%, especially 10% compared with the activity before mutation. Accordingly, in the case of the mutant α-amylases of the present invention obtained by such amino acid mutations, a fermented concentrate solution of a high concentration is available at a high yield and enzyme formulation treatment such as ultrafiltration after fermentation production can be conducted efficiently.

A combination of two or more substitutions or deletions of various amino acid residues is also effective for such amino acid mutations. It is also possible to use the above-exemplified mutation in combination with a mutation for improving enzymatic properties, for example, in an α-amylase having an amino acid sequence represented by SEQ ID No. 1 or having at least 60% homology thereto, a mutation for improving heat resistance by deleting amino acid residues corresponding to Arg_{181} and Gly_{182}, a mutation for improving oxidant resistance by substituting the amino acid residue corresponding to Met_{222} with Thr or a mutation for improving solubility by substituting the amino acid residue corresponding Lys_{484} with Gln; or in an α-amylase having an amino acid sequence represented by SEQ ID No. 2 or having at least 60% homology thereto, a mutation for further reinforcing oxidant resistance by substituting the

amino acid residue corresponding to Met_{107} with Leu or a mutation for heightening detergency of a laundry detergent by substituting the amino acid residue corresponding Glu_{188} with Ile.

A mutant α-amylase is available at a high yield by appropriately combining a mutant α-amylase structural gene with a control gene and a proper plasmid vector, thereby constructing a plasmid for the production of the α-amylase, introducing the resulting plasmid into a microorganism such as *Bacillus subtilis* or *Escherichia coli*, preferably, *Bacillus subtilis* and cultivating the resulting recombinant microorganism.

The mutant α-amylase thus obtained has improved productivity by about 10 to 500% as shown later in Examples while maintaining biochemical properties as an enzyme, thus having excellent properties. By the above-described mutation of the amino acid residues of liquefying alkaline α-amylases having heat resistance, chelating agent resistance, oxidant resistance and high solubility, it is therefore possible to create useful enzymes having drastically improved productivity in a recombinant microorganism without losing the above-described original properties.

The detergent compositions of the present invention may contain, in addition to the α-amylase of the invention,

one or more than one enzymes selected from debranching enzymes (such as pullulanase, isoamylase and neopullulanase), α-glucosidase, glucoamylase, protease, cellulase, lypase, pectinase, protopectinase, pectate lyase, peroxidase, laccase and catalase.

The detergent composition may contain, in addition, components ordinarily added to a detergent, for example, surfactants such as anionic surfactants, amphoteric surfactants, nonionic surfactants and cationic surfactants, chelating agents, alkali agents, inorganic salts, anti-redeposition agents, chlorine scavengers, reducing agents, bleaching agents, fluorescent dye solubilizing agents, perfumes, anti-caking agents, enzyme activating agents, antioxidants, antiseptics, blueing agents, bleach activating agents, enzyme stabilizing agents and regulator.

The detergent compositions of the invention can be produced in a manner known per se in the art from a combination of the highly productive α-amylase available by the above-described method and the above-described known detergent components. The form of the detergent can be selected according to the using purpose and examples include liquid, powder and granule. The detergent compositions of the present invention are suited as laundry detergents, bleaching detergents, detergents for automatic dish washer, pipe cleaners, and artificial tooth cleaners,

of which they are especially suited as laundry detergents, bleaching detergents and detergents for automatic dish washer.

The highly productive mutant α-amylases of the invention are also usable as starch liquefying · saccharifying compositions. Moreover, these mutant α-amylases, after addition thereto of one or more than one enzymes selected from glucoamylase, maltase, pullulanase, isoamylase and neopullulanase, can be allowed to act on starches.

Furthermore, the mutant α-amylases of the present invention are usable as a desizing composition of fibers and an enzyme such as pullulanase, isoamylase or neopullulanase can be incorporated in the composition.

Examples

Measurement of amylase activity and protein content

Amylase activity and protein content of the enzymes each produced from recombinant *Bacillus subtilis* were measured in accordance with the below-described methods.

Amylase activity was measured by the 3,5-dinitrosalicylic acid method (DNS method). After reaction at 50°C for 15 minutes in a reaction mixture of a 40 mM glycine - sodium hydroxide buffer (pH 10) containing soluble starch, the reducing sugar thus formed was

quantitatively analyzed by the DNS method. As the titer of the enzyme, the amount of the enzyme which formed reducing sugar equivalent to 1 μmol of glucose in one minute was defined as one unit.

The protein content was determined by "Protein Assay Kit" (product of Bio-Rad Laboratories) using bovine serum albumin as standard.

Referential Example 1: Screening of liquefying alkaline amylase

About 0.5 g of soil was suspended in sterilized water and the resulting suspension was heat treated at 80°C for 15 minutes. The supernatant of the heat treated mixture was diluted with an adequate amount of sterilized water, followed by applying to an isolating agar medium (Medium A). The medium was then cultured at 30°C for 2 days to grow colonies. The colonies which formed transparent zones in their peripheries due to starch dissolution were selected and isolated as amylase producing strains. The resulting isolated strains were inoculated in Medium B, followed by aerobic shaken culture at 30°C for 2 days. After cultivation, the chelating agent (EDTA) resisting capacity of the supernatant obtained by centrifugation was measured and in addition, the optimum working pH was measured. Thus, strain *Bacillus* sp. KSM-K38 (FERM BP-6946) was obtained.

Medium A: Tryptone 1.5%

	Soytone	0.5%
	Sodium chloride	0.5%
	Colored starch	0.5%
	Agar	1.5%
	Na_2Co_3	0.5%
	(pH 10)	
Medium B:	Tryptone	1.5%
	Soytone	0.5%
	Sodium chloride	0.5%
	Soluble starch	1.0%
	Na_2CO_3	0.5%
	(pH 10)	

The mycological properties of strain KSM-K38 are shown in Table 1.

Table 1

	Strain KSM-K38
(a) Observation under microscope	Cells are rods of a size of 1.0 to 1.2 μm × 2.4 to 5.4 μm in the strain K36 and 1.0 to 1.2 μm × 1.8 to 3.8 μm in the strain K38, and form an elliptical endospore (1.0 to 1.2 μm × 1.2 to 1.4 μm) at their subterminals or center. They have flagella and are motile. Gram's staining is positive. Acid fastness: negative.
(b) Growth in various culture mediums. The strains are alikaliphilic so that 0.5% sodium carbonate was added to the culture medium in the tests described hereinafter.	
· Nutrient agar plate culture	Growth of cells is good. Colony has a circular shape, with its surface being smooth and its peripheral end being smooth. The color of the colony is yellowish brown.
· Nutrient agar slant culture	Cells can grow.
· Nutrient broth	Cells can grow.
· Stab culture in nutrient-broth gelatin	Growth of cells is good. Liquefaction of gelatin is not observed.
· Litmus milk medium	No change in growth.
(c) Physiological properties	
· Nitrate reduction and denitrification	Nitrate reduction: positive Denitrification: negative
· MR test	Indeterminable because the medium is an alkaline medium.
· V-P test	Negative
· Production of indole	Negative
· Production of hydrogen sulfide	Negative
· Hydrolysis of starch	Positive
· Utilization of citric acid	Positive in Christensen's medium but negative in Koser's medium and Simmon's medium.
· Utilization of inorganic nitrogen sources	Nitrate is utilized but ammonium salts are not.
· Production of colorants	Negative
· Urease	Negative
· Oxidase	Negative
· Catalase	Positive
· Growth range	Growth temperature range: 15 to 40 °C, optimum growth temperature: 30 °C, growth pH range: pH 9.0 to 11.0, optimum growth pH range: same
· Behavior on oxygen	Aerobic
· O-F test	Cells do not grow
· Sugar utilization	L-galactose, D-xylose, L-arabinose, lactose, glycerin, melibiose, ribose, D-glucose, D-mannose, maltose, sucrose, trehalose, D-mannitol, starch, raffinose and D-fructose are utilized.
Growth in a salt-containing medium	Cells can grow when salt concentration is 12%, but not when salt concentration is 15%.

Referential Example 2: Cultivation of strain KSM-K38

In the liquid medium B of Referential Example 1, the strain KSM-K38 was inoculated, followed by aerobic shaken culture at 30°C for 2 days. The amylase activity (at pH 8.5) of each of the supernatants isolated by centrifugation was measured. As a result, the activity in 1 L of the culture medium was found to be 1177 U.

Referential Example 3: Purification of liquefying alkaline amylase

Ammonium sulfate was added to the supernatant of the culture medium of the strain KSM-K38 obtained in Referential Example 2 to give 80% saturation, followed by stirring. The precipitate thus formed was collected and dissolved in a 10mM tris-HCl buffer (pH 7.5) containing 2 mM $CaCl_2$ to dialyze the resulting solution against the buffer overnight. The dialysate was loaded on a DEAE-Toyopearl 650M column equilibrated with the same buffer and protein was eluted in a linear gradient of 0 to 1 M of NaCl in the same buffer. The active fraction obtained by gel filtration column chromatography after dialysis against the same buffer was dialyzed against the buffer, whereby purified enzyme exhibited a single band on polyacrylamide gel electrophoresis (gel concentration: 10%) and sodium dodecylsulfate (SDS) electrophoresis was obtained.

Referential Example 4: Enzymological properties

The properties of the purified enzyme are as follows:

(1) Action

It acts on starch, amylose, amylopectin and α-1,4-glycoside bond which is a partially degraded product thereof to degrade them and produce, from amylose, glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6) and maltoheptaose (G7). But it does not act on pullulan.

(2) pH Stability (Britton-Robinson buffer)

It exhibits residual activity of 70% or more within a range of pH 6.5 to 11.0 under treating conditions at 40°C for 30 minutes.

(3) Working temperature range and optimum working temperature

It acts in a wide temperature range of from 20 to 80°C, with the optimum working temperature being 50 to 60°C.

(4) Temperature stability

The temperature at which the enzyme loses its activity was examined by causing a temperature change in a 50 mM glycine - sodium hydroxide buffer (pH 10) and then, treating at each temperature for 30 minutes. The residual activity of the enzyme is 80% or more at 40°C and about 60% even at 45°C.

(5) Molecular weight

The molecular weight as measured by sodium-dodecylsulfate polyacrylamide gel electrophoresis is 55,000

± 5,000.

(6) Isoelectric point

Its isoelectric point as measured by isoelectric focusing electrophoresis is about 4.2.

(7) Effects of surfactants

It is almost free from activity inhibition (activity remaining ratio: 90% or more) even when treated at pH 10 and 30°C for 30 minutes in a 0.1% solution of a surfactant such as sodium linear alkylbenzene sulfonate, alkyl sulfate ester sodium salt, polyoxyethylene alkylsulfate ester sodium salt, sodium α-olefin sulfonate, sodium α-sulfonated fatty acid ester, sodium alkylsulfonate, SDS, soap and softanol.

(8) Effects of metal salts

It was treated at pH 10 and 30°C for 30 minutes in each of the reaction systems containing various metal salts and their effects were studied. Its activity is inhibited by 1 mM of Mn^{2+} (inhibition ratio: about 75%) and slightly inhibited by 1 mM of Sr^{2+} and Cd^{2+} (inhibition ratio: about 30%).

Example 1: <u>Preparation of various recombinant plasmids having an α-amylase gene ligated thereto</u>

In accordance with the method as described in WO98/44126, genes encoding a mutant α-amylase (which will hereinafter be described as "ΔRG") having improved heat

resistance and a mutant α-amylase ("ΔRG-M202T") having improved oxidant resistance as well as improved heat resistance were constructed, respectively, by deleting Arg_{181} and Gly_{182} of the α-amylase ("LAMY") which was derived from the strain *Bacillus* sp. KSM-AP1378 (FERM BP-3048) and had the amino acid sequence represented by SEQ ID No. 1; and by, in addition to this mutation by deletion, substituting Thr for Met_{202} of the amino acid sequence represented by SEQ ID No. 1. With the genes as a template, gene fragments (about 1.5 kb) encoding these mutant α-amylases were amplified by the PCR reaction using primers LAUS (SEQ ID No. 5) and LADH (SEQ ID No. 6). After cutting of them with a restriction enzyme SalI, each of the fragments was inserted into the SalI-SmaI site of an expression vector pHSP64 (Japanese Patent Application Laid-Open No. Hei 6-217781), whereby a recombinant plasmid having a structural gene of each of the mutant α-amylases bonded thereto was constructed downstream of a strong promoter derived from an alkaline cellulase gene of the strain *Bacillus* sp. KSM-64 (FERM P-10482).

In the meantime, with a chromosomal DNA, which had been extracted from the cells of the strain *Bacillus* sp. KSM-K38 (FERM BP-6946) by the method of Saito and Miura (Biochim. Biophys. Acta, **72**, 619(1961)), as a template, PCR reaction was effected using primers K38US (SEQ ID No. 7)

and K38DH (SEQ ID No. 8) shown in Table 2, whereby a structural gene fragment (about 1.5kb) encoding an α-amylase (which will hereinafter be described as "K38AMY") having an amino acid sequence of SEQ ID No. 2 was amplified. After cutting of it with a restriction enzyme SalI, the resulting fragment was inserted into the SalI-SmaI site of an expression vector pHSP64 to construct, downstream of a strong promoter derived from an alkaline cellulase gene of the strain *Bacillus* sp. KSM-64 (FERM P-10482) contained in pHSP64, a recombinant plasmid having a structural gene of K38AMY bonded thereto (FIG. 1). With this recombinant plasmid as a template, PCR reaction was effected using the primers CLUBG (SEQ ID. No. 9) and K38DH (SEQ. ID. 8) to amplify a gene fragment (about 2.1 kb) having the strong promoter and K38AMY bonded thereto.

By the recombinant PCR method as described below, a gene encoding chimeric α-amylase between K38AMY and LAMY was constructed. Described specifically, with a chromosomal DNA of the strain KSM-K38 (FERM BP6946) as a template, PCR reaction was conducted using primers K38DH (SEQ ID No. 8) and LA-K38 (SEQ ID No. 10) shown in Table 2, whereby a fragment encoding the sequence from Gln_{20} downstream to the C-terminal of the amino acid sequence of K38AMY represented by SEQ ID No. 2 was amplified. With the above-described recombinant plasmid containing the LAMY

gene and strong promoter as a template, PCR reaction was conducted using primers CLUBG (SEQ ID No. 9) and LA-K38R (SEQ ID No. 11) shown in Table 2, whereby a gene fragment encoding from the upstream strong promoter to Gly_{21} of the amino acid sequence of LAMY of SEQ ID No. 1 was amplified. By the second PCR reaction using the resulting two DNA fragments and primers CLUBG (SEQ ID No. 9) and K38DH (SEQ ID No. 8) shown in Table 2, the resulting two fragments having, at the end thereof, complementary sequences derived from primers LA-K38 (SEQ ID No. 10) and LA-K38R (SEQ ID No. 11) respectively were combined, whereby a gene fragment (about 2.1kb) encoding a chimeric α-amylase (which will hereinafter be described as "LA-K38AMY") which has, successively bonded thereto, a region encoding from His_1 to Gly_{21} of the LAMY downstream of the strong promoter and a region encoding from Gln_{20} to the C-terminal of the K38AMY was amplified.

By using a "Site-Directed Mutagenesis System Mutan-Super Express Km" kit (product of Takara Shuzo Co., Ltd.), the below-described mutations were introduced to the K38AMY and LA-K38AMY. First, the K38AMY and LA-K38AMY gene fragments (about 2.1kb) were inserted into the site SmaI of a plasmid vector pKF19k attached to the kit to construct a mutagenic recombinant plasmid (FIG. 2). A site-directed mutagenic oligonucleotide primer N190F (SEQ ID No. 50)

shown in Table 2 was 5'-phosphorylated with T4 DNA kinase. Using this and the above-described mutagenic recombinant plasmid, mutagenesis was effected in accordance with the method of the kit and by using the reaction product, the strain *Escherichia coli* MV1184 ("Competent cell MV1184", product of Takara Shuzo Co., Ltd.) was transformed. From the transformant thus obtained, a recombinant plasmid was extracted, followed by analysis of a basic sequence, whereby mutation by substitution of Phe for Asn_{190} was confirmed. By repeated introduction of mutagenic reactions into the mutated gene by successively using primers A209V (SEQ ID No. 51) and QEYK (SEQ ID No. 49) in a similar manner as above, thereby substituting Asn_{190} and Gln_{209}, each of the amino acid sequence of the K38AMY represented by SEQ ID No. 2, with Phe and Val, respectively, and the sequence from Asp_1 to Gly_{19} of the amino acid sequence of the K38AMY represented by SEQ ID No. 2 with the sequence from His_1 to Gly_{21} of the amino acid sequence of the LAMY represented by SEQ ID NO. 1; by substituting Gln_{167}, Tyr_{169}, Asn_{190} and Gln_{209}, each of the amino acid sequence of the K38AMY, with Glu, Lys, Phe and Val, respectively and the sequence from Asp_1 to Gly_{19} of the amino acid sequence of the K38AMY with the sequence from His_1 to Gly_{21} of the amino acid sequence of the LAMY; and substituting Gln_{167} and Tyr_{169}, Asn_{190} and Gln_{209}, each of the amino acid sequence of the K38AMY, with Glu, Lys, Phe and Val, respectively, genes encoding a

mutant α-amylase (which will hereinafter be described as "LA-K38AMY/NFQV") having improved heat resistance, a mutant α-amylase ("LA-K38AMY/QEYK/NFQV") having drastically improved heat resistance, and a mutant α-amylase ("QEYK/NFQV") having improved heat resistance were constructed, respectively.

With these genes as a template, PCR reaction was conducted using primers K38US (SEQ ID No. 7) and K38DH (SEQ ID No. 8) to amplify structural gene fragments (about 1.5kb) encoding the mutant α-amylases were amplified. They were then inserted into the SalI-SmaI site of an expression vector pHSP64 in a similar manner as above, whereby a recombinant plasmid having structural genes of these mutant α-amylases bonded each other was constructed (FIG. 1).

Example 2: Introduction of a mutation for improving α-amylase productivity

A "Site-Directed Mutagenesis System Mutan-Super Express Km" kit of Takara Shuzo Co., Ltd. was used for site-directed mutagenesis for improving amylase productivity of recombinant *Bacillus subtilis*. With various recombinant plasmids obtained in Example 1 as a template, PCR reactions were effected using primers CLUBG (SEQ ID No. 9) and LADH (SEQ ID No. 6) for ΔRG and ΔRG/M202T, while using primers CLUBG (SEQ ID No. 9) and

K38DH (SEQ ID No. 8) for K38AMY, LA-K38AMY/NFQV, LA-K38AMY/QEYK/NFQV and QEYK/NFQV, whereby fragments of about 2.1kb from the upstream strong promoter derived from the strain KSM-64 to the downstream α-amylase gene were amplified. These amplified fragments were inserted into the SmaI site of a plasmid vector pKF19k attached to the above-described kit, whereby various mutagenetic recombinant plasmids were constructed (FIG. 2).

Various oligonucleotide primers for site-directed mutagenesis shown in Table 2 (SEQ ID Nos. 12 to 51) were 5'-phosphorylated with T4DNA kinase, and by using the resultant products and the above mutagenetic recombinant plasmids, mutagenesis was conducted in accordance with the method as described in the kit. With the reaction products, the strain *Escherichia coli* MV1184 ("Competent Cell MV1184" product of Takara Shuzo Co., Ltd.) was transformed. From the resulting transformants, a recombinant plasmid was extracted, followed by analysis of a base sequence to confirm mutation.

Table 2

SeQ ID No.	Primer	Base sequence (5'-3')	Using purpose
5	LAUS	GAGTCGACCAGCACAAGCCCATCATAATGG	PCR for recombi-nation
6	LADH	TAAAGCTTCAATTTATATTGG	
7	K38US	GGGTCGACCAGCACAAGCCGATGGATTGAACGGTACGATG	
8	K38DH	TAAAGCTTTTGTTATTGGTTCACGTACAC	
9	CLUBG	CCAGATCTACTTACCATTTTAGAGTCA	
10	LA-K38	ATTTGCCAAATGACGGGCAGCATTGGAATCGGTT	
11	LA-K38R	AACCGATTCCAATGCTGCCCGTCATTTGGCAAAT	
12	P18S	TTTGAATGGCATTTGTCAAATGACGGGGAACCAC	Site-directed mutagenesis (ΔRG)
13	Q86E	ACAAGGAGTCAGTTGGAAGGTGCCGTGACATCT	
14	E130V	CGAAACCAAGTAATATCAGGT	
15	N154D	AATACCCATTCCGATTTTAAATGGCGC	
16	R171C	GATTGGGATCAGTCATGYCAGCTTCAGAACAAA	
17	A186V	AAATTCACCGGAAAGGTATGGGACTGGGAAGTA	
18	E212D	TCATCCAGATGTAATCAATG	
19	V222E	CTTAGAAATTGGGGAGAATGGTATACAAATACA	
20	Y243C	GTGAAACATATTAAATGCAGCTATACGAGAGAT	
21	P260E	AACACCACAGGTAAAGAAATGTTTGCAGTTGCA	
22	K269Q	AGAATTTTGGCAAAATGACCT	
23	E276H	TTGCTGCAATCCATAACTATTTAAAT	
24	N277S	CTTGCTGCAATCGAAAGYTATTTAAATAAAACA	
25	R310A	GGCTATTTTGATATGGCAAATATTTTAAATGGT	
26	E360Q	TCTGACAAGGCAGCAAGGTTA	
27	Q391E	GATCCACTTCTGGAAGCACGTCAAACG	
28	W439R	GGGGGTAATAAAAGAATGTATGTCGGG	
29	K444R	ATGTATGTCGGGCGACATAAAGCTGG	
30	N471D	GATGGTTGGGGGGATTTCACTGTAA	
31	G476D	TTCACTGTAAACGATGGGGCAGTTTCG	
32	K484Q	GGTTTGGGTGCAGCAATAAAT	
33	P18X	TTTGAATGGCATTTGNNNAATGACGGGAACCAC	Site-directed mutagenesis (for ΔRG/ M2027)
34	A186X	AAATTCACCGGAAAGNNNTGGGACTGGGAAGTA	
35	Y243X	GTGAAACATATTAAANNNAGCTATACGAGAGAT	
36	N277X	CTTGCTGCAATCGAANNNTATTTAAATAAAACA	
37	N471E	GATGGTTGGGGGGAATTCACTGTAA	
38	D128V	CCAACGAATCGTTGGCAGGTAATTTCAGGTGCCTACACG	Site-directed mutagenesis (for K38AMY)
39	G140S	ATTGATGCGTGGACGAGTTTCGACTTTTCAGGG	
40	S144P	TTTCGACTTTCCAGGGCGTAA	
41	R168Q	GGTGTTGACTGGGATCAGCAATATCAAGAAAATCATATTTTCC	
42	N181V	CATATTTTCCGCTTTGCAAATACGGTNTGGAACAGGCGAGTG	
43	E207D	AATATCGACTTTAGTCATCCAGATGTACAAGATGAGTTGAAGGA	
44	F272S	GACGTAGGTGCTCTCGAATCTTATTTAGATGAAATGAATTGGG	
45	S375P	CGATAACATTCCAGCTAAAAA	
46	W434R	GACCTGGTGGTTCCAAGAGAATGTATGTAGGACGTCAG	
47	E466D	AATGGCGATGGATGGGGCGATTTCTTTACGAATGGAGGATCT	
48	D128X	CCAACGAATCGTTGGCAGNNNATTTCAGGTGCCTACACG	
49	QEYK	GTTGACTGGGATGAGCGCAAACAAGAAAATCAT	
50	N190F	TGGATGAAGAGTTCGGTAATTATGA	
51	Q209	AGTCATCCAGAGGTCGTAGATGAGTTGAAGGAT	

The "N" in the base sequence means a mixed base of A, T, G and C, while "Y" means a mixed base of T and C.

By inserting an expression promoter region and the mutant α-amylase gene portion into the SmaI site of pKF19k again in a similar manner as the above, the mutation-introduced gene became a template plasmid upon introduction of another mutation. Another mutation was thus introduced in a similar manner to the above-described method.

With these mutated recombinant plasmids thus obtained as a template, PCR reaction was conducted using primers CLUBG (SEQ ID No. 9) and LADH (SEQ ID No. 6) or primers CLUBS (SEQ ID No. 9) and K38DH (SEQ ID No. 8) to amplify the mutated gene fragments. After they were cut with SalI, they were inserted into the site of SalI-SmaI site of an expression vector pHSP64, whereby various plasmids for producing mutant α-amylases were constructed (FIG. 1).

Example 3: Production of mutant α-amylases

The various plasmids for producing mutant α-amylases obtained in Example 2 were each introduced into the strain *Bacillus subtilis* ISW1214 (leuA metB5 hsdM1) in accordance with the protoplast method. The recombinant *Bacillus subtilis* thus obtained was cultivated at 30°C for 4 days in a liquid medium (corn steep liquor, 4%; tryptose, 1%; meet extract, 1%, monopotassium phosphate, 0.1%, magnesium sulfate, 0.01%, maltose, 2%, calcium chloride, 0.1%, tetracycline, 15 μg/mL). The activity of each of the various mutant α-amylases was measured using the

supernatant of the culture medium.

Example 4: Evaluation of amylase productivity - 1

Each of an enzyme having Pro_{18} of ΔRG substituted with Ser (which will hereinafter be abbreviated as "P18S/ΔRG"), an enzyme having Gln_{86} substituted with Glu ("Q86E/ΔRG"), an enzyme having Glu_{130} substituted with Val ("E130V/ΔRG"), an enzyme having Asn_{154} substituted with Asp ("N154D/ΔRG"), an enzyme having Arg_{171} substituted with Cys ("R171C/ΔRG"), an enzyme having Ala_{186} substituted with Val ("A186V/ΔRG"), an enzyme having Glu_{212} substituted with Asp ("E212D/ΔRG"), an enzyme having Val_{222} substituted with Glu ("V222E/ΔRG"), an enzyme having Tyr_{243} substituted with Cys ("Y243C/ΔRG"), an enzyme having Pro_{260} substituted with Glu ("P260E/ΔRG"), an enzyme having Lys_{269} substituted with Gln ("K269E/ΔRG"), an enzyme having Glu_{276} substituted with His ("E276H/ΔRG"), an enzyme having Asn_{277} substituted with Ser ("N277S/ΔRG"), an enzyme having Arg_{310} substituted with Ala ("R310A/ΔRG"), an enzyme having Glu_{360} substituted with Gln ("E360Q/ΔRG"), an enzyme having Gln_{391} substituted with Glu ("Q391E/ΔRG"), an enzyme having Trp_{439} substituted with Arg ("W439R/ΔRG"), an enzyme having Lys_{444} substituted with Arg ("K444R/ΔRG"), an enzyme having Asn_{471} substituted with Asp ("N471D/ΔRG"),

and an enzyme having Gly_{476} substituted with Asp ("G476D/ΔRG) was assayed for amylase productivity. As a control, ΔRG was employed. A relative value (%) of amylase productivity was determined from the amylase productivity of ΔRG set at 100%. The results are shown in Table 3.

Table 3

Enzyme	Relative amylase productivity (%)
ΔRG	100
P18S/ΔRG	277
Q86E/ΔRG	119
E130V/ΔRG	362
N154D/ΔRG	146
R171C/ΔRG	235
A186V/ΔRG	485
E212D/ΔRG	327
V222E/ΔRG	135
Y243C/ΔRG	350
P260E/ΔRG	142
K269Q/ΔRG	142
E276H/ΔRG	231
N277S/ΔRG	312
R310A/ΔRG	208
E360Q/ΔRG	162
Q391E/ΔRG	127
W439R/ΔRG	312
K444R/ΔRG	112
N471D/ΔRG	292
G476D/ΔRG	296

Any one of the mutant enzymes exhibited higher amylase productivity than ΔRG, indicating that mutation heightened productivity of α-amylase in recombinant *Bacillus subtilis*. In particular, the productivity of each of E130V/ΔRG,

A186V/ΔRG, E212D/ΔRG, Y243C/ΔRG, N277S/ΔRG and W439R/ΔRG was found to be at least 3 times greater than that of ΔRG and above all, A186V/ΔRG exhibited eminently high productivity of almost 5 times greater than that of ΔRG.

Example 5: Evaluation of amylase productivity - 2

In a similar manner to the methods described in Examples 1, 2 and 3, each of an enzyme having Pro_{18} of ΔRG/MT substituted with Thr (which will hereinafter be abbreviated as "P18T/ΔRG/MT"), an enzyme having Gln_{86} substituted with Glu ("Q86E/ΔRG/MT"), an enzyme having Glu_{130} substituted with Val ("E130V/ΔRG/MT"), an enzyme having Ala_{186} substituted with Asn ("A186N/ΔRG/MT"), an enzyme having Tyr_{243} substituted with Ser ("Y243S/ΔRG/MT"), an enzyme having Asn_{277} substituted with Phe ("N277F/ΔRG/MT), and an enzyme having Asn_{471} substituted with Glu ("N471E/ΔRG/MT") was assayed for amylase productivity. As a control, ΔRG/MT was employed. The results are shown in Table 4.

Table 4

Enzyme	Relative amylase productivity (%)
ΔRG/MT	100
P18T/ΔRG/MT	200
Q86E/ΔRG/MT	144
E130V/ΔRG/MT	344
A186N/ΔRG/MT	344
Y243S/ΔRG/MT	189
N277F/ΔRG/MT	256
N471E/ΔRG/MT	211

It was recognized that any one of the above-described mutant enzymes exhibited high amylase productivity compared with ΔRG/MT, and in particular, the productivity of each of E130V/ΔRG/MT and A186N/ΔRG/MT was at least 3 times greater than that of ΔRG/MT.

Example 6: Evaluation of amylase productivity - 3

In accordance with the methods employed in Examples 1, 2 and 3, each of an enzyme having Asp_{128} of K38AMY substituted with Val (which will hereinafter be abbreviated as "D128V"), an enzyme having Gly_{140} substituted with Ser ("G140S"), an enzyme having Ser_{144} substituted with Pro ("S144P"), an enzyme having Arg_{168} substituted with Gln ("R168Q"), an enzyme having Asn_{181} substituted with Val ("N181V"), an enzyme having Glu_{207} substituted with Asp ("E207D"), an enzyme having Phe_{272} substituted with Ser ("F272S"), an enzyme having Ser_{375} substituted with Pro ("S375P"), an enzyme having Trp_{434} substituted with Arg

("W434R"), and an enzyme having Glu_{466} substituted with Asp ("E466D") was assayed for amylase productivity. As a control, K38AMY was employed. The results are shown in Table 5.

Table 5

Enzyme	Relative amylase productivity (%)
K38AMY	100
D128V	325
G140S	209
S144P	197
R168Q	264
N181V	207
E207D	109
F272S	175
S375P	115
W434R	124
E466D	212

It was recognized that compared with the wild type K38AMY, any one of the mutant enzymes exhibited high amylase productivity and in particular, D128V exhibited high productivity at least 3 times greater than that of K38AMY.

Example 7: Evaluation of amylase productivity - 4

A mutant enzyme S144P/N181V (which will hereinafter be abbreviated as "SPNV") having, among the mutants shown in Example 6, S144P and N181V in combination was assayed for amylase productivity in accordance with the method as described in Example 3. As a control, K38AMY, S144P and N181V were employed. The results are shown in Table 6.

Table 6

Enzyme	Relative amylase productivity (%)
K38AMY	100
S144P	197
N181V	207
SPNV	257

As a result, as shown in Table 6, a further improvement in amylase productivity was brought about by combined use.

Example 8: Evaluation of amylase productivity - 5

In accordance with the methods as described in Examples 1, 2 and 3, each of an enzyme obtained by substituting Arg_{168} of the gene of a heat-resistance improved enzyme LA-K38AMY/NFQV with Gln (which will hereinafter be abbreviated as "R168Q/LA-K38AMY/NFQV"), an enzyme obtained by substituting Glu_{466} of the above-described gene with Asp ("E466D/LA-K38AMY/NFQV"), and an enzyme having double mutations of Example 6 introduced into the gene ("SPNV/LA-K38AMY/NFQV") was assayed for amylase productivity. As a control, LA-K38AMY/NFQV was employed. The results are shown in Table 7.

Table 7

Enzyme	Relative amylase productivity (%)
LA-K38AMY/NFQV	100
R168Q/LA-K38AMY/NFQV	304
E466D/LA-K38AMY/NFQV	264
SPNV/LA-K38AMY/NFQV	154

As a result, it was recognized that any one of the mutant enzymes obtained in this Example exhibited high amylase productivity at least about 1.5 times greater than that of LA-K38AMY/NFQV and in particular, R168Q/LA-K38AMY/NFQV exhibited about 3 times greater productivity.

Example 9: Evaluation of amylase productivity - 6

In accordance with the methods as described in Examples 1, 2 and 3, each of an enzyme obtained by substituting Asp_{128} of the gene of a heat-resistance improved enzyme LA-K38AMY/QEYK/NFQV with Val (which will hereinafter be abbreviated as "D128V/LA-K38AMY/QEYK/NFQV") and an enzyme having double mutations of Example 6 introduced into the gene ("SPNV/LA-K38AMY/QEYK/NFQV") was assayed for amylase productivity. As a control, LA-K38AMY/QEYK/NFQV was employed. The results are shown in Table 8.

Table 8

Enzyme	Relative amylase productivity (%)
LA-K38AMY/QEYK/NFQV	100
D128V/LA-K38AMY/QEYK/NFQV	602
SPNV/LA-K38AMY/QEYK/NFQV	427

As a result, it was recognized that any one of the mutant enzymes obtained in this Example exhibited markedly high amylase productivity compared with LA-K38AMY/QEYK/NFQV and in particular, D128V/LA-K38AMY/QEYK/NFQV exhibited drastic increase (about 6 times) in productivity.

Example 10: Evaluation of amylase productivity - 7

Into D128V/LA-K38AMY/QEYK/NFQV which was recognized to show a drastic increase in productivity among the mutant enzymes shown in Example 9, a mutation for heightening oxidant resistance by substituting Met_{107} with Leu (this mutation will hereinafter be abbreviated as "M107L") was introduced in accordance with the methods as described in Examples 1 and 2 ("ML/DV/LA-K38AMY/QEYK/NFQV").

Then, the gene of the mutant enzyme ML/DV/LA-K38AMY/QEYK/NFQV was assayed for amylase productivity in accordance with the method of Example 4. As a control, D128V/LA-K38AMY/QEYK/NFQV was employed. The results are shown in Table 9.

Table 9

Enzyme	Relative amylase productivity (%)
D128V/LA-K38AMY/QEYK/NFQV	100
M107L/D128V/LA-K38AMY/QEYK/NFQV	115

The relative amylase productivity of the mutant enzyme ML/DV/LA-K38AMY/QEYK/NFQV was 115%, indicating that introduction of M107L mutation for reinforcing oxidant resistance did not adversely affect high productivity of amylase in recombinant *Bacillus subtilis*.

Example 11: Evaluation of amylase productivity - 8

In accordance with the methods as described in Examples 1, 2 and 3, an enzyme obtained by substituting

Asp_{128} of the gene of heat-resistance-improved enzyme QEYK/NFQV with Gln (the resultant enzyme will hereinafter be abbreviated as "D128Q/QEYK/NFQV") was assayed for amylase productivity. As a control, QEYK/NFQV was employed. The results are shown in Table 10.

Table 10

Enzyme	Relative amylase productivity (%)
QEYK/NFQV	100
D128Q/QEYK/NFQV	247

It was recognized that the mutant enzyme exhibited productivity of at least 2 times greater than that of QEYK/NFQV.

Example 12: Solubility assay

After storage of each of the mutant enzyme preparations as shown in Table 11 at 4°C for 1 week, the precipitate formed by centrifugation (13000 rpm, 10 minutes, 4°C) was separated. The precipitate was suspended in the same volume, as that before centrifugation, of a Tris-HCl buffer (pH 7.0) containing of 2 mM $CaCl_2$. The resulting suspension was diluted about 500-folds with the same buffer to dissolve the former in the latter and enzymatic activity in the resulting solution was measured. The supernatant was diluted in a similar manner and enzymatic activity in it was also measured. Solubility of each of the mutant enzymes was evaluated by comparing the

enzymatic activity in each of the precipitate solution and supernatant with that of the preparation before storage at 4°C. The results are shown collectively in Table 11.

Table 11

Enzyme	Residual activity (%) after storage at 4°C	
	Supernatant	Precipitate
ΔRG	55	40
ΔRG Gln86 → Glu	83	11
ΔRG Pro260 → Glu	70	18
ΔRG Lys269 → Gln	74	27
ΔRG Asn471 → Asp	74	23
ΔRG Lys484 → Gln	71	24

As a result, when an improved α-amylase (ΔRG) having heat resistance improved by deleting Arg_{181} and Gly_{182} was stored at 4°C for one week, precipitation of the enzyme was recognized and only about half of the activity remained in the supernatant. On the other hand, the mutant enzymes obtained by introducing a further mutation in ΔRG-LAMY showed a high activity residual ratio in the supernatant, indicating an improvement in solubility by mutation. In particular, the enzyme having Gln_{86} substituted with Glu showed the highest enzyme solubility and 80% of the enzyme remained in the supernatant under the conditions of this Example.

Example 13: Detergent composition for automatic dish washer

A detergent composition for automatic dish washer having the composition as shown in Table 12 was prepared,

followed by incorporation therein of various mutant enzymes obtained in the productivity increasing method. As a result, the highly productive mutant enzymes exhibited similar or superior detergency to the control enzyme when they were equal in activity.

Table 12

Composition of detergent	(%)
Pluronic L-61	2.2
Sodium carbonate	24.7
Sodium bicarbonate	24.7
Sodium percarbonate	10.0
No. 1 sodium silicate	12.0
Trisodium citrate	20.0
Polypropylene glycol	2.2
"Silicone KST-04" (product of Toshiba Silicone)	0.2
"Sokalan CP-45" (product of BASF)	4.0

Capability of Exploitation industry

By using the mutant α-amylases according to the present invention, α-amylases are available at a high yield from recombinant microorganisms, making it possible to largely reduce the cost of their industrial production. The mutation for productivity increase in the present invention does not adversely affect biochemical properties of the enzymes so that highly productive liquefying alkaline α-amylases having heat resistance, chelating agent resistance and oxidant resistance and being useful as enzymes for a detergent can be produced.

SEQUENCE LISTING

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ttg tac aat gca tct aat agt ggt ggc tat ttt gat atg aga aat att 1183

Leu Tyr Asn Ala Ser Asn Ser Gly Gly Tyr Phe Asp Met Arg Asn Ile

300 305 310

tta aat ggt tct gtc gta caa aaa cac cct ata cat gca gtc aca ttt 1231

Leu Asn Gly Ser Val Val Gln Lys His Pro Ile His Ala Val Thr Phe

315 320 325

gtt gat aac cat gac tct cag cca gga gaa gca ttg gaa tcc ttt gtt 1279

Val Asp Asn His Asp Ser Gln Pro Gly Glu Ala Leu Glu Ser Phe Val

330 335 340

caa tcg tgg ttc aaa cca ctg gca tat gca ttg att ctg aca agg gag 1327

Gln Ser Trp Phe Lys Pro Leu Ala Tyr Ala Leu Ile Leu Thr Arg Glu

345 350 355 360

caa ggt tac cct tcc gta ttt tac ggt gat tac tac ggt ata cca act 1375

Gln Gly Tyr Pro Ser Val Phe Tyr Gly Asp Tyr Tyr Gly Ile Pro Thr

365 370 375

cat ggt gtt cct tcg atg aaa tct aaa att gat cca ctt ctg cag gca 1423

His Gly Val Pro Ser Met Lys Ser Lys Ile Asp Pro Leu Leu Gln Ala

380 385 390

cgt caa acg tat gcc tac gga acc caa cat gat tat ttt gat cat cat 1471
Arg Gln Thr Tyr Ala Tyr Gly Thr Gln His Asp Tyr Phe Asp His His
395 400 405

gat att atc ggc tgg acg aga gaa ggg gac agc tcc cac cca aat tca 1519
Asp Ile Ile Gly Trp Thr Arg Glu Gly Asp Ser Ser His Pro Asn Ser
410 415 420

gga ctt gca act att atg tcc gat ggg cca ggg ggt aat aaa tgg atg 1567
Gly Leu Ala Thr Ile Met Ser Asp Gly Pro Gly Gly Asn Lys Trp Met
425 430 435 440

tat gtc ggg aaa cat aaa gct ggc caa gta tgg aga gat atc acc gga 1615
Tyr Val Gly Lys His Lys Ala Gly Gln Val Trp Arg Asp Ile Thr Gly
445 450 455

aat agg tct ggt acc gtc acc att aat gca gat ggt tgg ggg aat ttc 1663
Asn Arg Ser Gly Thr Val Thr Ile Asn Ala Asp Gly Trp Gly Asn Phe
460 465 470

act gta aac gga ggg gca gtt tcg gtt tgg gtg aag caa taaataagga 1712
Thr Val Asn Gly Gly Ala Val Ser Val Trp Val Lys Gln
475 480 485

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ttatgatata tgtaagcgtt atcattaaaa ggaggtattt g atg aga aga tgg gta 176

gta gca atg ttg gca gtg tta ttt tta ttt cct tcg gta gta gtt gca 224

gat gga ttg aac ggt acg atg atg cag tat tat gag tgg cat ttg gaa 272

Asp Gly Leu Asn Gly Thr Met Met Gln Tyr Tyr Glu Trp His Leu Glu

1 5 10 15

aac gac ggg cag cat tgg aat cgg ttg cac gat gat gcc gca gct ttg 320

Asn Asp Gly Gln His Trp Asn Arg Leu His Asp Asp Ala Ala Ala Leu

20 25 30

agt gat gct ggt att aca gct att tgg att ccg cca gcc tac aaa ggt 368

Ser Asp Ala Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Tyr Lys Gly

35 40 45

aat agt cag gcg gat gtt ggg tac ggt gca tac gat ctt tat gat tta 416

Asn Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu

50 55 60

gga gag ttc aat caa aag ggt act gtt cga acg aaa tac gga act aag 464

Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys

65 70 75 80

gca cag ctt gaa cga gct att ggg tcc ctt aaa tct aat gat atc aat 512

Ala Gln Leu Glu Arg Ala Ile Gly Ser Leu Lys Ser Asn Asp Ile Asn

85 90 95

gta tac gga gat gtc gtg atg aat cat aaa atg gga gct gat ttt acg 560

Val Tyr Gly Asp Val Val Met Asn His Lys Met Gly Ala Asp Phe Thr

100 105 110

gag gca gtg caa gct gtt caa gta aat cca acg aat cgt tgg cag gat 608

Glu Ala Val Gln Ala Val Gln Val Asn Pro Thr Asn Arg Trp Gln Asp

115 120 125

att tca ggt gcc tac acg att gat gcg tgg acg ggt ttc gac ttt tca 656

Ile Ser Gly Ala Tyr Thr Ile Asp Ala Trp Thr Gly Phe Asp Phe Ser

130 135 140

ggg cgt aac aac gcc tat tca gat ttt aag tgg aga tgg ttc cat ttt 704

Gly Arg Asn Asn Ala Tyr Ser Asp Phe Lys Trp Arg Trp Phe His Phe

145 150 155 160

aat ggt gtt gac tgg gat cag cgc tat caa gaa aat cat att ttc cgc 752

Asn Gly Val Asp Trp Asp Gln Arg Tyr Gln Glu Asn His Ile Phe Arg

165 170 175

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Phe Ala Asn Thr Asn Trp Asn Trp Arg Val Asp Glu Glu Asn Gly Asn

180 185 190

tat gat tac ctg tta gga tcg aat atc gac ttt agt cat cca gaa gta 848
Tyr Asp Tyr Leu Leu Gly Ser Asn Ile Asp Phe Ser His Pro Glu Val
195 200 205

caa gat gag ttg aag gat tgg ggt agc tgg ttt acc gat gag tta gat 896
Gln Asp Glu Leu Lys Asp Trp Gly Ser Trp Phe Thr Asp Glu Leu Asp
210 215 220

ttg gat ggt tat cgt tta gat gct att aaa cat att cca ttc tgg tat 944
Leu Asp Gly Tyr Arg Leu Asp Ala Ile Lys His Ile Pro Phe Trp Tyr
225 230 235 240

aca tct gat tgg gtt cgg cat cag cgc aac gaa gca gat caa gat tta 992
Thr Ser Asp Trp Val Arg His Gln Arg Asn Glu Ala Asp Gln Asp Leu
245 250 255

ttt gtc gta ggg gaa tat tgg aag gat gac gta ggt gct ctc gaa ttt 1040
Phe Val Val Gly Glu Tyr Trp Lys Asp Asp Val Gly Ala Leu Glu Phe
260 265 270

tat tta gat gaa atg aat tgg gag atg tct cta ttc gat gtt cca ctt 1088
Tyr Leu Asp Glu Met Asn Trp Glu Met Ser Leu Phe Asp Val Pro Leu
275 280 285

aat tat aat ttt tac cgg gct tca caa caa ggt gga agc tat gat atg 1136
Asn Tyr Asn Phe Tyr Arg Ala Ser Gln Gln Gly Gly Ser Tyr Asp Met
290 295 300

cgt aat att tta cga gga tct tta gta gaa gcg cat ccg atg cat gca 1184
Arg Asn Ile Leu Arg Gly Ser Leu Val Glu Ala His Pro Met His Ala
305 310 315 320

gtt acg ttt gtt gat aat cat gat act cag cca ggg gag tca tta gag 1232
Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Glu Ser Leu Glu

325 330 335

tca tgg gtt gct gat tgg ttt aag cca ctt gct tat gcg aca att ttg 1280
Ser Trp Val Ala Asp Trp Phe Lys Pro Leu Ala Tyr Ala Thr Ile Leu

340 345 350

acg cgt gaa ggt ggt tat cca aat gta ttt tac ggt gat tac tat ggg 1328
Thr Arg Glu Gly Gly Tyr Pro Asn Val Phe Tyr Gly Asp Tyr Tyr Gly

355 360 365

att cct aac gat aac att tca gct aaa aaa gat atg att gat gag ctg 1376
Ile Pro Asn Asp Asn Ile Ser Ala Lys Lys Asp Met Ile Asp Glu Leu

370 375 380

ctt gat gca cgt caa aat tac gca tat ggc acg cag cat gac tat ttt 1424
Leu Asp Ala Arg Gln Asn Tyr Ala Tyr Gly Thr Gln His Asp Tyr Phe

385 390 395 400

gat cat tgg gat gtt gta gga tgg act agg gaa gga tct tcc tcc aga 1472
Asp His Trp Asp Val Val Gly Trp Thr Arg Glu Gly Ser Ser Ser Arg

405 410 415

cct aat tca ggc ctt gcg act att atg tcg aat gga cct ggt ggt tcc 1520
Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asn Gly Pro Gly Gly Ser

420 425 430

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Lys Trp Met Tyr Val Gly Arg Gln Asn Ala Gly Gln Thr Trp Thr Asp

435 440 445

tta act ggt aat aac gga gcg tcc gtt aca att aat ggc gat gga tgg 1616
Leu Thr Gly Asn Asn Gly Ala Ser Val Thr Ile Asn Gly Asp Gly Trp

450 455 460

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Gly Glu Phe Phe Thr Asn Gly Gly Ser Val Ser Val Tyr Val Asn Gln

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